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CATABOLIC REPRESSION OF BACTERIAL SPORULATION

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The formation of endospores in bacteria is generally studied in media which do not support growth: the exhausted growth medium or a solution of mineral salts into which washed growing bacteria are transferred.[1, 2] Under carefully adjusted cultural conditions, a reasonably synchronous sporulation can thus be obtained in the majority of the population. There seems therefore to be no need, and even no possibility, for a change in the conventional methods used to study sporulation.

An entirely new approach became available in 1961, however, when it was observed that spores are constantly being formed in cultures of *Bacillus megaterium* growing exponentially in a mineral-glucose medium.[3] Similar results have recently been obtained with *B. subtilis*.[4] Relative to the total viable population, the number of spores first reaches a limit value, and then increases with time at a constant rate, equal to the growth rate of the total population.[3]

The present work investigates the influence of various factors on the size of the sporulated fraction (hereafter called ρ) in growing cultures of *B. subtilis*. The interpretation of the results is that the probability for a cell in a growth medium to become committed to sporulate[1, 5] must be determined by the intracellular concentration of at least one nitrogen-containing catabolite repressing, directly or indirectly, the expression of all the sporulation genes. A preliminary account of this work has been included in recent reviews;[6, 7] further evidence supporting the proposed interpretation will be published elsewhere.[8, 9]

Experimental Procedure.—The wild Marburg strain of *Bacillus subtilis* has been used. A common mineral base for all media was prepared from the following three solutions (all figures in gm/liter); *solution I:* K_2HPO_4, 10.5; KH_2PO_4, 3.5; *solution II:* $MgSO_4$, $7H_2O$, 5.0; $FeSO_4$, $7H_2O$, 0.5; *solution III:* $CaCl_2$, 5.0; $MnCl_2$, $4H_2O$, 0.5; solutions I and III were autoclaved, solution II sterile-filtered. The base medium is made of 100 vol of solution I for 1 vol each of solutions II and III. Various substances are then added as carbon or as nitrogen source; the complete medium is thus fully described with the mention of the nature of each source, followed, in gm/liter, by its concentration [e.g., NH_4Cl(0.5)-glucose(2)].

A complex medium also has been used: Difco nutrient broth, 8.0; KCl, 1.0; $MgSO_4$, $7H_2O$, 0.25; $MnCl_2$, $4H_2O$, 0.002. After adjusting the pH to 7.0 and autoclaving, sterile $CaCl_2$ and $FeSO_4$ were added to 5×10^{-4} M and 1×10^{-6} M, respectively. Solidified with agar (17 gm/liter), it was used for plating. After 2 days of incubation, wild-type colonies are deep brown on this medium, the spores becoming pigmented; asporogenous mutant colonies remain white.[10] Thus, if such mutants were selected in the experiments to be described, their selection could not escape attention when colonies were counted.

Stock cultures in NH_4Cl-glucose medium were kept at $-40°C$ in the presence of glycerol (15%). *Precultures:* From a thawed stock culture a series of tenfold dilutions was made in the selected medium. The cultures were shaken at 37°C; before the last dilution tube reached maximum turbidity, a new series of dilutions was made from it. No preculture was used to inoculate an experimental culture unless at least 15 generations had taken place in the medium under study. When this is done, the measured value for ρ is stable. *Experimental cultures and counting:* These cultures were inoculated from a growing preculture and shaken at 37°C, unless indicated otherwise. Turbidity measurements, viable counts, and spore counts were made at intervals during exponential growth. No difficulty due to chain formation was encountered, since daughter bacteria separate readily in the simple media employed. Spore counts were made after heating a sample of culture for 10 min at 80°C, in sealed ampoules immersed in a water bath. As a rule, several hundred colonies were counted in each count.

Theoretical Considerations and Presentation of the Results.—During exponential growth the total bacterial population (N_T) is made up of three kinds of cells: the growing cells (N_G); the cells committed to sporulate (N_C), the sporangial parts of which are assumed not to grow; and the thermoresistant spores (N_S). We shall call μ the growth rate, in doublings per hour, ρ the sporulated fraction (i.e., N_S/N_T), π the probability for a cell to become committed to sporulate, and τ the time required for a committed cell to reach thermoresistance.

The values of these parameters are not easily derived from the viable counts made on the culture before and after heat treatment. The "unheated" count is an overestimated value for N_G and an underestimated one for N_T, since unheated spores do not germinate quantitatively. The nongrowing fraction being small, however, this count is taken as a measure of N_T. The "heated" count expresses the number of spores present at the time of treatment. What we really want to measure, however, is the number of spores formed in the culture up to that time; spore germination must therefore be taken into account. The required correction has been made by introducing the factor α, i.e., the percentage of germination of unheated spores in the medium under consideration. The values of α in three different media have been measured, using spores that had just formed in complex medium, were not liberated yet from their sporangium, and had not been heat-activated. The values found for α are 0.75 in the casein-hydrolysate(10)–NH_4Cl(0.5)–glucose(2) medium, 0.45 in the NH_4Cl(0.5)–glucose(2) medium, and 0.25 in the NH_4Cl(0.5)–citrate(2) medium. (In all three media, germination occurred during the first 30 min of incubation, the nongerminating spores remaining constant in number for at least 7 hr afterwards.) Based on these experimental figures, α values that seemed reasonable were arbitrarily assigned to the other media mentioned in Table 1, i.e., 0.75 to the NH_4Cl-glucose medium supplemented with L-alanine, 0.45 to all glucose-containing media, and 0.25 to all other media.

No ways were found to measure the parameter τ directly in the growing cultures; it has been assumed to be constant and the same as in an exhausted complex medium, i.e., *ca.* 7 hr at 37°C and 13 hr at 25°C. Some justification for doing so will be published elsewhere.

The mathematical analysis of the behavior of a population which is sporulating while growing leads for π to a complex expression, which, once equilibrium has been reached, can be reduced to the following equation:

$$\pi = \frac{\rho}{1 - \alpha} 2^{\mu\tau}. \quad (1)$$

The slope of the straight line expressing—in semilogarithmic coordinates—the increase with time of N_S or N_T is taken as a measure of μ. In each set of experimental conditions the value of π can thus be calculated from equation (1), the measured values of μ and ρ, and the adopted values for α and τ. We realize that the uncertainty concerning the actual values of α and τ is such that the values given for π can at best be approximations. Since π is the variable we really

want to study, however, we feel that values, even approximate, for this parameter are more revealing than accurate values of ρ, a complex variable with no obvious physiological meaning.

Results.—(1) *The influence of the nature of the carbon or the nitrogen source:* Curves representing growth (N_T) and sporulation during growth (N_S) in various media are reported in Figure 1; the parallel increase of these two variables can be seen in every medium tested. The experimental values of μ and ρ, together with the derived values of π, for six different carbon and nitrogen sources are presented in Table 1. It can be seen that the probability for a cell to become committed in a synthetic medium supporting growth is related to the composition of the medium. With NH_4Cl as the nitrogen source, π is of the order of 10 per cent with most of the carbon sources tried, but it is much smaller when malate is the substrate. In the presence of glucose, π varies within very wide limits, being negligible when all amino acids are supplied, and approaching unity when histidine is the only nitrogen source. It also appears that π is not directly related to the growth rate.

In the NH_4Cl medium, several carbon sources fail to support growth when given singly: lactose, galactose, xylose, 2-deoxyglucose, α-ketoglutarate, succinate, oxalacetate, acetate, and glyoxylate; in the glucose medium the same is true of the following nitrogen sources: L-phenylalanine, L-tyrosine, L-lysine, glycine, sodium nitrite, and formamide. The cases of pyruvate, L-histidine (given as a carbon source), and sodium nitrate are complicated by the rapid selection of sporulation mutants and will be dealt with in another publication.[9]

(2) *The influence of the concentration of the carbon and energy source:* The effect of varying the concentration of the carbon source is presented in Table 2. In the range of concentration studied, μ, as expected, is concentration-independent. The value of π, however, is seen, with two different carbon sources, to vary within very wide limits.

(3) *The influence of temperature:* The relative rates of synthesis of the various macromolecular components of the cell are known to be dependent on the composition of the growth medium, but independent of temperature.[11, 12] Since a Q_{10} of *ca.* 2 is expected on the growth rate in a given medium when the temperature is varied between 25 and 37°C, it was of interest to determine the values of ρ and π in various

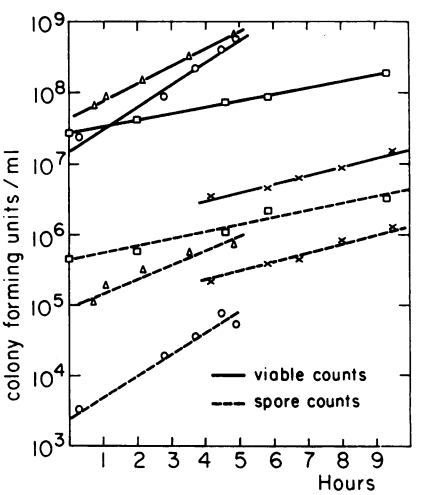

FIG. 1.—Growth and sporulation during growth of *B. subtilis* Marburg at 37°C in various minimal media. By plating on complex agar medium, two counts are made at various times from the experimental cultures (see *Procedures*): a viable count on the unheated sample, and a spore count after heat treatment. O, NH_4Cl(0.5)–glucose(2) medium; △, NH_4Cl(0.5)–ribose(2) medium; □, NH_4Cl(0.5)–citrate(2) medium; ×, L-histidine(1.5)–glucose(2) medium.

TABLE 1

GROWTH AND SPORULATION DURING GROWTH IN VARIOUS SYNTHETIC MEDIA AT 37°C

A. Varying the nature of the carbon source in the NH_4Cl(0.5) medium

Carbon source (2 gm/liter)	μ (doublings/hr)	ρ	π
Glucose	0.9	3×10^{-4}	5×10^{-2}
Malate	0.8	1×10^{-6}	6×10^{-5}
Glycerol	0.8	2×10^{-3}	13×10^{-2}
Ribose	0.8	2×10^{-3}	13×10^{-2}
Lactate	0.4	1×10^{-2}	9×10^{-2}
Citrate	0.3	2×10^{-2}	11×10^{-2}

B. Varying the nature of the nitrogen source in the glucose(2) medium

Nitrogen source (gm/liter)	μ (doublings/hr)	ρ	π
Casein hydrolysate (1)	1.7	$<2 \times 10^{-9}$	$<3 \times 10^{-5}$
L-alanine (1)	1.0	5×10^{-4}	25×10^{-2}
NH_4Cl (0.5)	0.9	3×10^{-4}	5×10^{-2}
L-aspartate (1)	0.65	7×10^{-5}	4×10^{-3}
L-glutamate (1)	0.5	$1\text{–}10 \times 10^{-3}$*	$2.5\text{–}25 \times 10^{-2}$*
L-histidine (1)	0.4	8×10^{-2}	

* Partial agglutination of the culture prevented an accurate determination.

TABLE 2

EFFECT ON GROWTH AND SPORULATION DURING GROWTH OF VARYING THE CONCENTRATION OF THE CARBON SOURCE*

A. Glucose is the only carbon source

Glucose (gm/liter)	μ (doublings/hr)	ρ	π
0.3	0.9	8×10^{-4}	14×10^{-2}
2	0.9	3×10^{-4}	5×10^{-2}
5	0.9	1×10^{-4}	1.5×10^{-2}
10	0.9	8×10^{-6}	1.5×10^{-3}
50	0.9	1×10^{-6}	1.5×10^{-4}

B. Glycerol is the only carbon source

Glycerol (gm/liter)	μ (doublings/hr)	ρ	π
2	0.85	2×10^{-3}	17×10^{-2}
50	0.85	6×10^{-5}	5×10^{-3}

* NH_4Cl (0.5) medium, incubation at 37°C.

TABLE 3

EFFECT OF TEMPERATURE ON GROWTH AND SPORULATION DURING GROWTH

Medium (gm/liter)	25°C μ (doublings/hr)	25°C ρ	25°C π	37°C μ (doublings/hr)	37°C ρ	37°C π
Nutrient broth (8)	0.9	$<2 \times 10^{-9}$	$<3 \times 10^{-5}$	2.0	$<2 \times 10^{-9}$	$<1 \times 10^{-3}$
Casein-hydrolysate (1)-glucose (2)	0.75	$<2 \times 10^{-9}$	$<6 \times 10^{-5}$	1.7	$<2 \times 10^{-9}$	$<3 \times 10^{-5}$
NH_4Cl(0.5)-glucose (2)	0.45	4×10^{-4}	4×10^{-2}	0.9	3×10^{-4}	5×10^{-2}
Glutamate (1)-glucose (2)	0.25	5×10^{-3}	10×10^{-2}	0.5	$1\text{–}10 \times 10^{-3}$*	$2.5\text{–}25 \times 10^{-2}$*

* Not accurately determined, the cultures being slightly agglutinated.

media at these two temperatures. The results of such experiments, given in Table 3, demonstrate, in every medium tested, the temperature independence of ρ and π. By changing the temperature, μ and π may vary independently. Particularly striking is the fact that the value of π for cells growing in the NH_4Cl-glucose medium at 37°C is at least 10^3 times greater than the corresponding value for cells growing in the casein hydrolysate–glucose medium at 25°C, in spite of a faster growth in the former medium.

(4) *Influence of various additions made to the NH_4Cl-glucose medium:* Determinations of μ and ρ have also been made in the NH_4Cl-glucose medium, to which various substances had been added singly at a concentration of 1 gm/liter; α has not been measured with these supplemented media, and the effect of these additions on ρ only will be reported. When equilibrium was reached, the sporulated fraction of the population was decreased by a factor of a least 50 in the presence of L-glutamate, L-leucine, L-aspartate, L-proline, DL-methionine, DL-malate, oxamate, or formamide. The effect was less pronounced with glycine, L-histidine, citrate, α ketoglutarate, succinate, and pyruvate; L-valine, D-glutamate, and acetate had no effect, even on growth rate, and growth was stopped when DL-serine or L-cysteine was added. In conclusion, even when added singly, several amino acids and intermediates of the tricarboxylic acid cycle will depress the measured value of ρ considerably, while increasing the growth rate by only a relatively small factor ($\leqslant 1.6$).

Discussion.—Catabolic repression of sporulation: The experiments reported clearly demonstrate the heterogeneity of exponentially growing bacterial populations with respect to their commitment to sporulate. The constancy of the sporulated fraction in such populations, after a period of adjustment, seems to indicate that every cell is faced, at some step at least of its growth cycle, with a choice between two mutually exclusive behaviors: to continue to grow or to sporulate. The probability with which the choice is made in favor of sporulation is shown to be relatively independent of the growth rate, but to depend on the nature and concentration of the carbon source being used. These observations strongly suggest that sporulation is repressed by catabolites, the internal concentrations of which would vary slightly from cell to cell.

The probability of sporulation during growth depends, in addition, on the nature of the available nitrogen source. As the case of histidase synthesis shows,[13] this is compatible with catabolic repression being at work. Only the enzymes which participate in carbohydrate metabolism exclusively have their synthesis repressed by ternary catabolites, which are formed whether a utilizable nitrogen source is present or not. But repressors of the enzymes participating in nitrogen metabolism are, in some cases at least, believed to contain nitrogen themselves.[14] That sporulation is repressed by nitrogen-containing catabolites seems therefore to be a tenable working hypothesis, which would explain, in addition, why sporulation during growth could not be discovered with *Bacillus* species which, like *B. cereus*, require several amino acids for growth.

Earlier observations made on bacilli sporulating *en masse* under conditions of starvation must be cited in this respect, although they seem to be contradictory. With *B. mycoides*, glucose, in the absence of nitrogen, has been reported to inhibit some early step(s) of spore formation, while ammonium salts released the inhibition.[15] With *B. megaterium*, however, mass sporulation occurs, whether the carbon *or* the

nitrogen source is lacking;[5] glucose is thus inhibitory only in the presence of a utilizable nitrogen source. Whatever the reason for these discrepancies (which seem to have escaped attention for the last 13 years), the repression in the Marburg strain of *B. subtilis* seems to be similar to the one described in *B. megaterium:* in the wild-type strains, repression occurs only when growth is possible.

Repression of enzyme synthesis is not an all-or-none phenomenon: its level reflects the intracellular concentration of the repressing metabolite,[16] which in turn depends on the rate of anabolic reactions as well as on the rate of repressor formation.[17] These notions apply to the repression of the whole process of spore formation, as the following observations demonstrate. (1) Some carbon or nitrogen sources, being more rapidly converted into repressor, should lead to a higher level of repression than could be anticipated from the growth rates. Such exceptional cases have been found: malate and aspartate seem to be especially good precursors of the repressor(s) under study (Table 1). (2) When in the NH_4Cl-glucose medium growth is limited by the rate of synthesis of the building blocks, a higher glucose concentration increases the repression level, leaving growth unaffected (Table 2). (3) If, as seems reasonable, the over-all catabolic and anabolic reactions have the same temperature coefficient, an increase in growth rate brought about by an elevation of temperature would not affect the repression level; this is precisely the case (Table 3). (4) When the synthesis of a given enzyme is under catabolic repression, the corresponding messenger RNA is apparently not formed.[18, 19] The formation of sporulation-specific messengers is therefore not expected to take place during growth and the mRNA fraction prepared from growing cells should not compete as effectively as the corresponding fraction from sporulating cells in hybridization tests made between denatured DNA and labeled sporulation messengers. This has recently been found to be the case.[20] The hypothesis under discussion seems therefore already supported by a variety of facts; other consequences derived from it, concerning the behavior of sporogenous strains grown in the chemostat, and the selection of superrepressed mutants, will be dealt with in separate publications.[8, 9]

The problem of the identification of the enzyme(s) repressed by catabolites: Catabolic repression, a feedback control mechanism, can only affect catabolic enzymes;[14] from this it is inferred that *derepression of at least one catabolic enzyme is necessary for sporulation to take place.* The problem then arises of the identification of this hypothetical key enzyme. A protease repressed by amino acids would appear as a possible candidate: some proteases are known to be repressed by amino acids,[21, 22] and others have been implicated in spore formation.[23–25] The notion of one key enzyme might be misleading, however, as the next section will show.

The problem of the coordinate repression of all sporulation genes: During the process of spore formation, at least six distinct cytological stages can be recognized,[26, 27] and many specific products are formed: the already-mentioned protease, antigens,[28, 29] lytic enzymes,[30–32] toxins,[33] antibiotics,[6, 34, 35] succinyl glutamic acid,[36] and the well-known dipicolinic acid.[37] Thus, judging from the phenotypic changes, many bacterial genes become expressed during the period of sporulation.

Sporulation mutants have been isolated and examined with the electron microscope;[26, 38, 39] they may be blocked at any one stage of the sporulation process. The Marburg strain being transformable[40] and transducible,[41] it should be possible

to build a map of these mutants. This is being done in several laboratories, and it already appears that the sporulation genes are numerous, and generally not linked;[10, 42] several unlinked clusters seem likely to exist.

Sporulation thus behaves as a long sequence of reactions catalyzed by enzymes having the common property of being repressed during growth. Since the whole sporulation process now seems to be repressed by catabolites, one must wonder whether the assumed nitrogen-containing catabolite(s) affect the functioning of all sporulation genes *directly*. If these genes belong in one and the same operon,[43] their apparently coordinate functioning would be easily accounted for, and no single sporulation enzyme would be in a key position. The absence of genetic linkage which seems to be the rule with randomly selected sporulation mutants and the widely different times at which the various products are known to appear are not in favor of this assumption, however. If, on the other hand, the sporulation genes are assumed to belong in several distinct operons, their apparently coordinated expression would be most easily explained by assuming that sequential induction[44] is at work. An enzyme of the first operon might be considered a key enzyme in this case. Some knowledge of the kind of clustering exhibited by the sporulation genes is obviously needed before the regulation mechanisms involved in sporulation can be analyzed further.

Summary.—In cultures of *B. subtilis* growing exponentially in minimal media, the numbers of spores and viable cells increase at the same rate, but the spore-to-cell ratio depends on the composition of the medium.[3] In a given medium the value of this ratio can be used to calculate the probability with which a bacterium will sporulate rather than multiply. This mathematical expression is believed to reflect the intracellular concentration of some nitrogen-containing catabolite(s), inasmuch as it depends on the nature of both the carbon *and the nitrogen* source present in the medium. The data are consistent with the hypothesis that such catabolites act as repressors of at least one sporulation-specific enzyme, which might be a protease.

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*STUDIES OF ADENOVIRUS-SV40 HYBRID VIRUSES, III. TRANSFER OF SV40 GENE BETWEEN ADENOVIRUS TYPES**†

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A number of adenovirus types have become "hybridized" with at least a portion of the SV40 viral genome as a consequence of prolonged passage in monkey kidney cultures contaminated with SV40 virus.[1-5] The adenovirus 7 (Ad. 7)-SV40 hybrid strain E46+ has been shown to consist of a mixture of nonhybrid adenovirus particles which are fully infectious for human embryonic kidney (HEK) cells in tissue culture but markedly limited in ability to propagate in African green monkey kidney (AGMK) cell cultures, and hybrid particles which are defective (i.e., single